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and Prostate Tumor Growth

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13. ABSTRACT (Maximum 200 Words) The objective of our research is to understand the molecular mechanism underlying the impact of various dietary components including fat, genistein, vitamin D, and selenium on prostate growth. Our research has demonstrated that the ventral prostate of the rats on high fat diet is 15% (p<0.001) heavier than that of the rats on low fat diet. The dietary influence on ventral prostate weight does not seem to involve androgen action axis because dietary components did not influence the expression of several androgen-response genes, serum testosterone (T) and dihydrotestosterone (DHT), and intraprostatic T and DHT in experimental animals. Thus, high fat diet is likely to modulate the ventral prostate weight via an androgen-independent mechanism. Using a highly sensitive PCR-based cDNA subtraction method, we have identified one gene that is down-regulated by dietary fat in the ventral prostate. Sequencing analysis revealed that this gene encodes prostatein C3, a ventral prostate-specific secretory protein. Dietary fat down-regulates the expression of prostatein C3 by 37% (p<0.05). Prostatein C3 represents the first example of genes that are regulated by dietary fat in the prostate <i>in vivo</i> . Our findings are potentially important because dietary fat is a major risk factor associated with prostate cancer incidence.		
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TABLE OF CONTENTS

Cover	1
Standard Form (SF) 298	2
Table of Contents	3
Introduction	4
Body	5-19
Key Research Accomplishments	19-20
Reportable Outcomes	20
Conclusions	20-21
References	21
Bibliography and Personnel Report:	22
Appendices	23-46

INTRODUCTION:

Nutrition is a major risk factor responsible for the differences in global distribution of clinical prostate cancer (Mandel and Schuman, 1980; Yatani et al., 1988). Western style diet, which consists of high fat content, is associated with a high rate of prostate cancer incidence. In contrast, Asia diet has low fat content and is rich in soy, which is associated with a low rate of prostate cancer incidence. Numerous human and animal studies showed that certain dietary ingredients could modulate the growth rate of prostate cancer cells. However, the mechanism by which dietary components impact on prostate cancer development and progression is not clear. An understanding at molecular level of dietary influence on prostate growth may lead to the development of novel approaches for preventing prostate cancer.

The objective of our research is to study the molecular mechanism of nutritional effect on normal and cancerous prostate growth. Our original hypothesis is that some nutritional ingredients can influence the expression of some androgen-response gene(s), which in turn could affect the proliferation rate of the prostate. The specific questions addressed or to be addressed in our research are following. Do nutritional ingredients influence prostate growth? If yes, which ingredient(s) could influence prostate growth? Do nutritional ingredients influence the androgen action pathway? If not, what is the molecular mechanism of dietary influence on prostate growth? Some of the above questions were addressed in our first year of the research in the rat model. The answers to the above questions will help us to prevent prostate cancer by modifying our diet or by developing new drugs that blocks fat action pathway.

BODY

Task 1. To determine the effect of dietary ingredients on androgen-response gene expression in the rat prostate (**month 1-30**).

A To study the effect of saturated fat and genistein on the expression of androgen-response genes in the rat prostate (**months 1-12**).

B. To study the effect of unsaturated fatty acids and vitamin D on the expression of androgen-response genes in the rat prostate (**months 6-18**).

To study the effect of the saturated fat on the expression of androgen-response genes, we initiated our experiments by feeding the rats with either high saturated fat diet (32.2% of calories from fat) or low fat diet (3.6% of calories from fat) for 1 month. Total RNA was extracted from the pooled prostate of the rats fed with the same diet.

Because the saturated fat did not affect the expression of androgen-response genes (See below), we decided to try to determine the effect of selenium, a mineral which has protective effect against the development of prostate cancer. Sprague-Dawley rats were fed either with selenium deficient or selenium surplus food for 1 month or 3 months before the prostates were isolated.

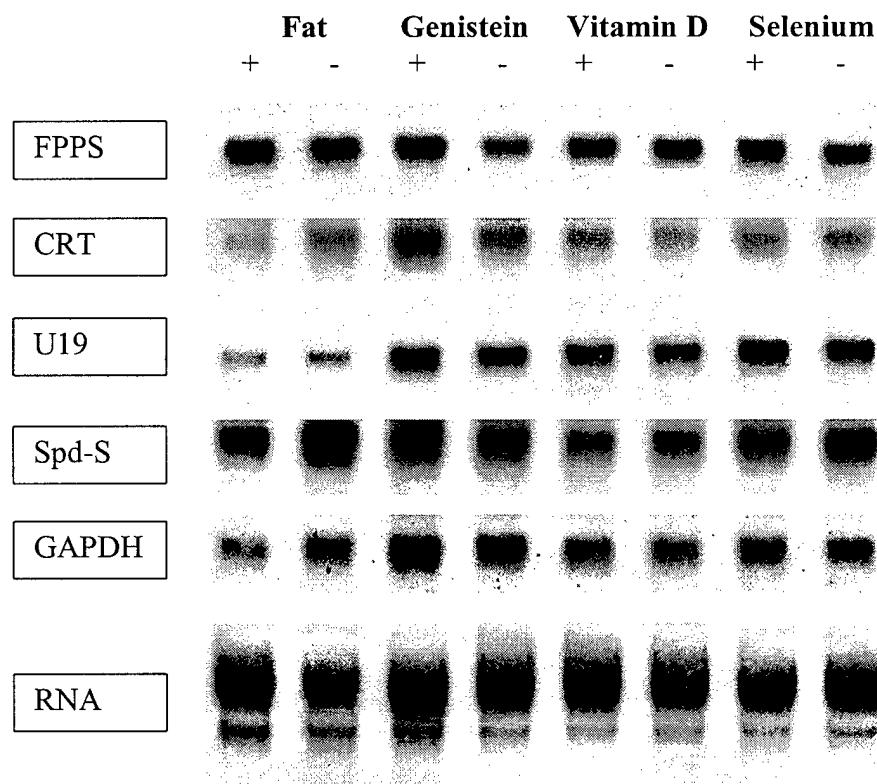


Fig. 1. Northern blot analysis of the ventral prostate of the rats fed with indicated pairs of diet for 1 month. The total RNA was extracted as previously described. The Northern blot was probed with indicated androgen-response genes. FPPS = Farnesyl Pyrophosphate Synthase; CRT =

Calreticulin; U19 = Up-regulated Gene 19 (no homology with known genes); Spd-S = Spermidine Synthase; GAPDH = Glyceraldehyde-3-phosphate dehydrogenase; RNA = Total RNA stained with methylene blue.

To study the effect of other dietary components on androgen-response genes, we have fed the animals 1 month with following food: genistein surplus (20 mg/kg food) and genistein deficient diet, vitamin D surplus (4,000 U/kg) or vitamin D deficient food, and selenium surplus (1.4 mg of Na₂SeO₃.5H₂O per kg) or selenium deficient food. The total RNA was extracted from the pooled left lobes of the ventral prostates of the rats fed with the same diet. The RNA was analyzed by Northern blot using cDNAs of various androgen-response genes as probes (Wang et al., 1997).

We have quantified the level of expression of various androgen-response genes by Phosphoimage. The Northern blot of FPPS, CRT, U19, Spd-S were shown in Fig. 1. GAPDH expression pattern and total RNA staining were included as controls for loading normalization. Our results indicate that the dietary components examined in our experiment have little or no influence on the expression of androgen response genes.

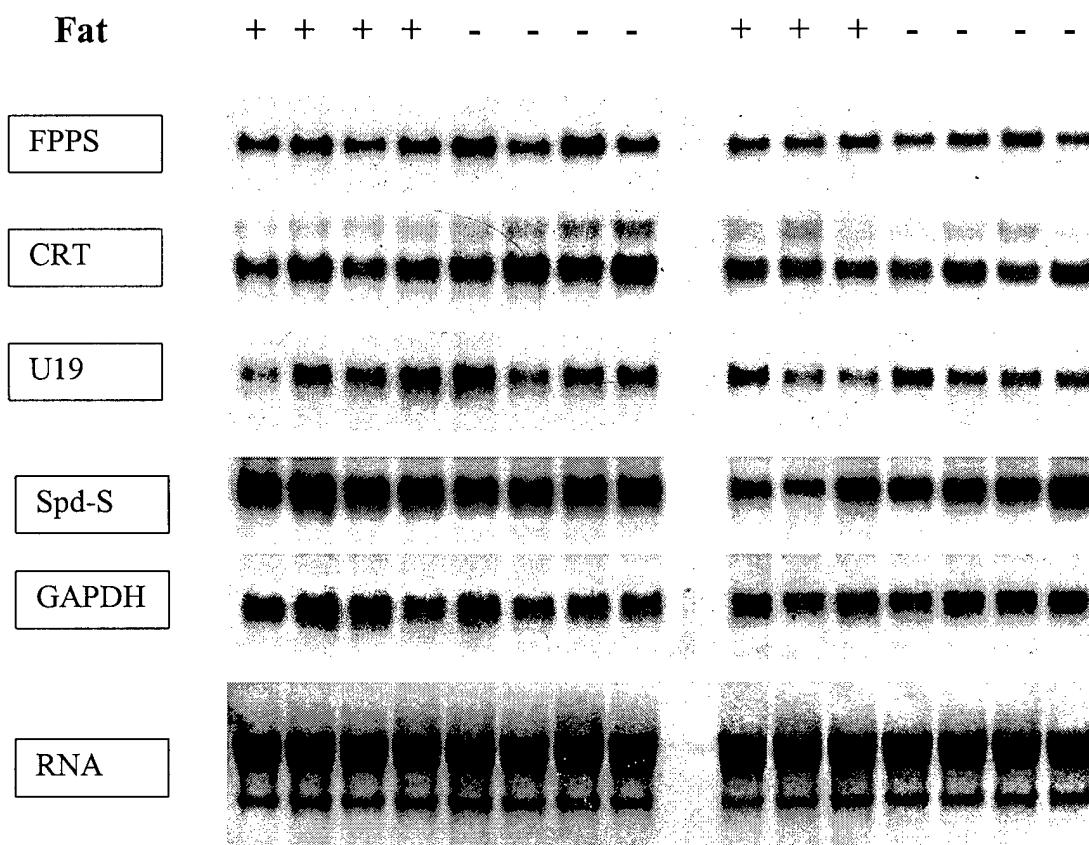


Fig. 2. Northern blot analysis of the ventral prostate of the rats fed with either with high fat diet (+) or low fat diet (-) for 3 months. The total RNA was extracted as previously described. The Northern blot was probed with indicated androgen-response genes. FPPS = Farnesyl Pyrophosphate Synthase; CRT = Calreticulin; U19 = Up-regulated Gene 19 (no homology with

known genes); Spd-S = Spermidine Synthase; GAPDH = Glyceraldehyde-3-phosphate dehydrogenase; RNA = Total RNA stained with methylene blue.

One exciting observation we have made in our present research is that the ventral prostate of the rats fed with high fat diet is larger than that of the rats fed with low fat diet (See below). Thus, we have isolated the individual rat left ventral prostate from the animals fed with either high fat or low fat diet for 3 months. The left lobes of the ventral prostate were isolated for total RNA extraction individually. The purified total RNA was analyzed by Northern blot to determine the expression of 2 dozen androgen-response genes (Wang et al., 1997). Examples of the Northern blot was shown in Fig. 2. The Northern blot signals were also quantified using phosphoimager. No significant alterations in the expression of androgen-response genes were detected (Results not shown). The differences in different lanes are due to loading variations.

C. To study the combinatorial effect of saturated fat, unsaturated fatty acids, vitamin D, and genistein on the expression of androgen-response genes in the rat prostate (**months 18-30**).

According to the proposed schedule, we have not carried out this task yet. Considering the fact that none of the dietary ingredients had any detectable effect on the expression of androgen-response genes, it is not necessary to conducting this task. Instead, as I suggested and requested in the section of **Recommended changes to better address the research topic**, we would like to study the mechanism by which high fat diet enhances the wet weight of the rat ventral prostate using a highly sensitive PCR-based cDNA subtraction method (Wang et al., 1997).

D. To analyze the collected raw data and samples (**months 1-30**). This will include measurement of serum T and DHT, calculation of animal caloric intake, measurement of body weight, measurement of wet weight and DNA content of the prostate, and Northern blot analysis of androgen-response gene expression in the prostate.

Data collection and analysis in this project are very time consuming. We have very carefully dissected the rat ventral prostate and collected serum. To maximize the use of animals, we have also taken a lot of effort to dissect the dorsal and lateral prostate and seminal vesicles. The dissection of dorsal and lateral prostate is very tedious and labor intensive. We have also weighed body weight and food consumed twice a week. The animal caloric intake, body weight, and wet weight of ventral, dorsal, and lateral prostate and seminal vesicles were analyzed and shown in the figures below.

The effect of saturated fat on the serum T and DHT was measured using the kits from Diagnostic Inc.. The serum T and DHT were measured in all animals individually. The results are interesting as revealed below.

In order to assay the intraprostatic T and DHT in every prostate individually, we have worked out a convenient method to accomplish this task. The details are being prepared in a short method paper for submission. The effect of saturated fat on the intraprostatic T and DHT was measured in the right lobe of the ventral prostate individually.

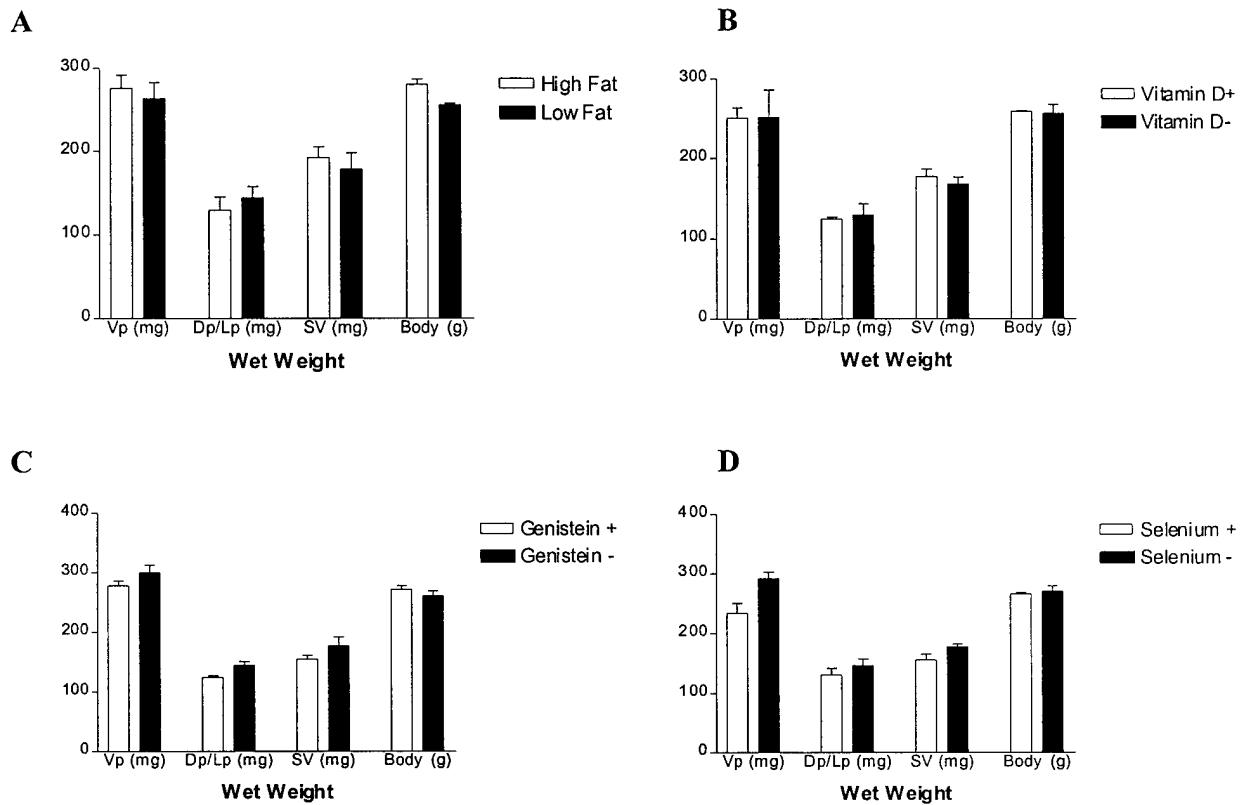


Fig. 3. The effect of high fat versus low fat (A), vitamin D surplus versus deficient (B), genistein surplus versus deficient (C), and selenium surplus versus deficient (D) on the wet weights of ventral prostate (Vp), dorsal/lateral prostate (Dp/Lp), and seminal vesicles (SV) and the body weight. Animals were fed with the indicated diet for 1 month. The feeding was initiated when the animals were 21 days old. The data were imported to the GraphPad software for analysis. The error bar represents standard error mean (SEM).

The result of Fig. 3A showed that the ventral prostate of the ventral prostate of the rats fed with high fat diet is slightly larger than that of the rats fed with low fat diet in 1 month feeding experiment. This result is reproducible in the 3 month feeding experiment (Fig. 4A).

Vitamin D had little or no effect on prostate size in this 1 month feeding experiment (Fig. 3B & 4B). One interesting observation of this experiment is that the size deviation of ventral prostate of the rats fed with vitamin D deficient diet is larger than the deviation in other groups. This suggests the importance of vitamin D in maintaining normal prostate structure and function.

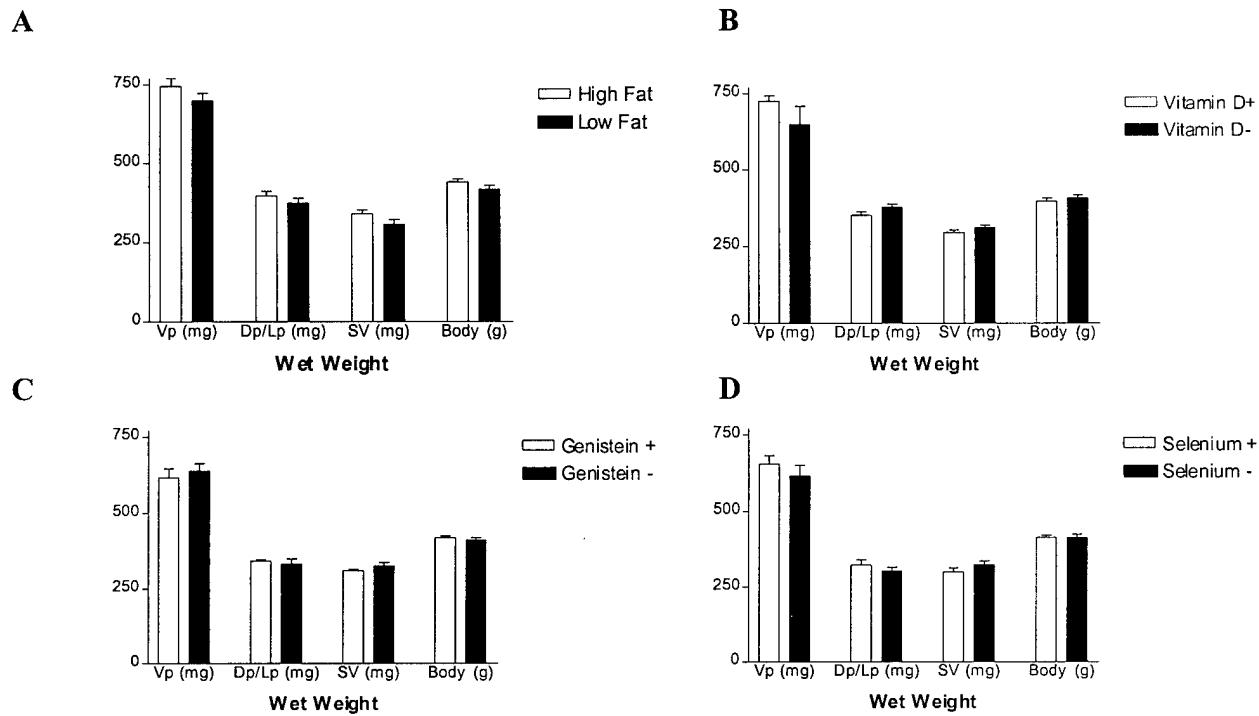


Fig. 4. The effect of high fat versus low fat (A), vitamin D surplus versus deficient (B), genistein surplus versus deficient (C), and selenium surplus versus deficient (D) on the wet weights of ventral prostate (Vp), dorsal/lateral prostate (Dp/Lp), and seminal vesicles (SV) and the body weight. Each group consists of 8 pairs of animals that were fed with the above diet for 3 month. The feeding was initiated when the animals were 21 days old. The data were imported to the GraphPad software for analysis. The error bar represents standard error mean (SEM).

Genistein appears to cause a small reduction of the size of the ventral prostate, dorsal and lateral prostate, and seminal vesicles. This suggests that genistein may influence androgen action in all male sex accessory organs (Fig. 3C). The small reduction in the size of ventral prostate by genistein is reproduced in the 3 month feeding experiment (Fig. 4C).

Selenium surplus appears to inhibit the ventral prostate significantly (Fig. 3D). However, this result is not reproducible in the 3 month feeding experiment (Fig. 4D). We addressed the possibility that selenium may inhibit prostate growth in 1-month feeding period. Additional feeding experiment indicates that the inhibition of ventral prostate growth in Fig. 1D is not reproducible (Results not shown).

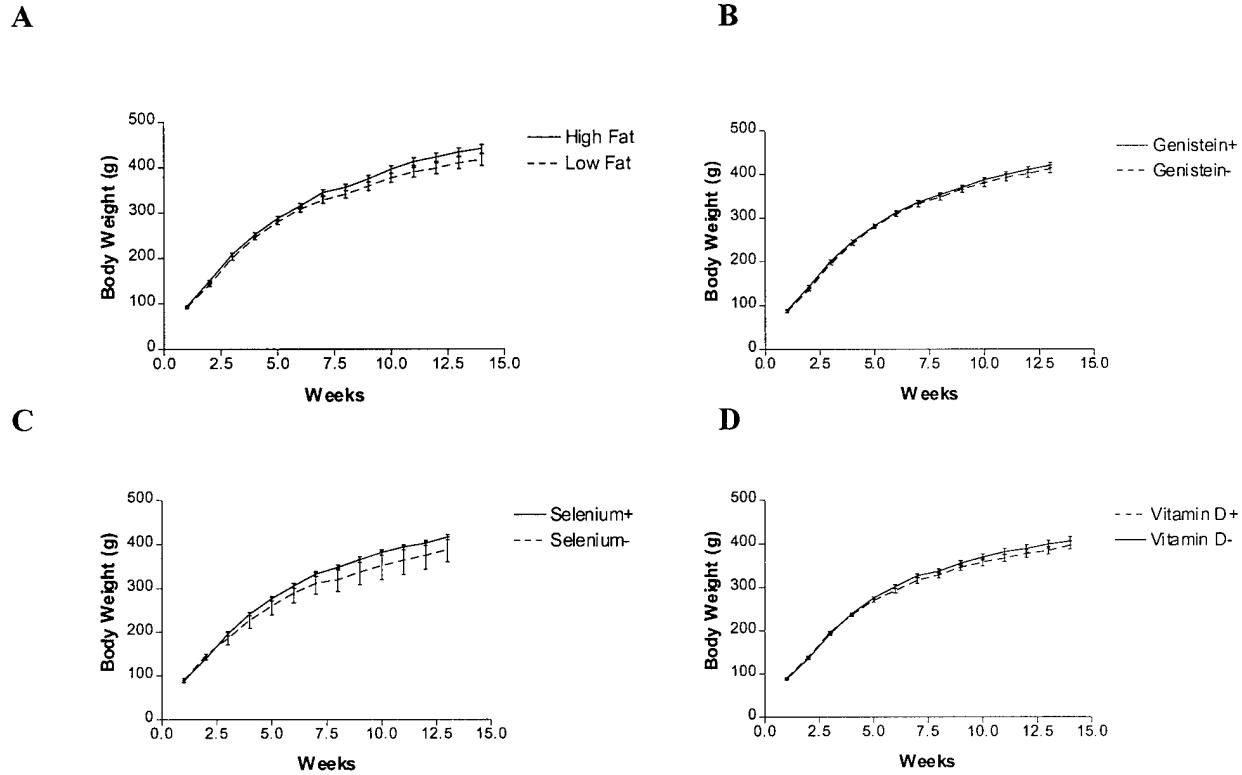
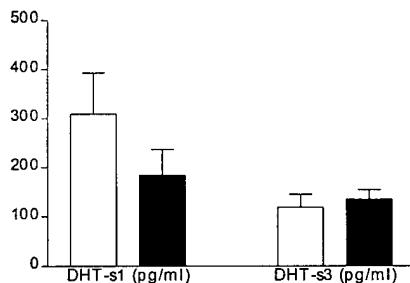


Fig. 5. The effect of high fat versus low fat (A), genistein surplus versus deficient (B), selenium surplus versus deficient (C), and vitamin D surplus versus deficient (D) on the growth of the rats.

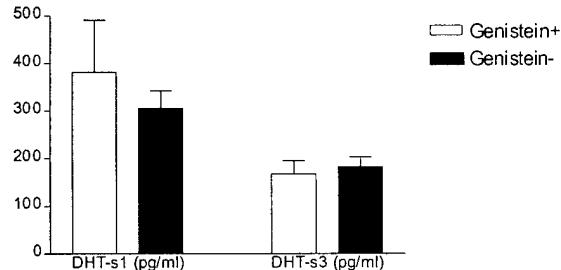
The rats fed with high fat diet is slightly heavier than the rats fed with low fat diet (Fig. 5A). The slight increase in body weight is most likely due to the accumulation of the fat in the body cavity of the animals because we can see that in the dissection.

Other dietary ingredients in our research do not influence the body weight. Genistein feeding does not influence the body weight of the animals (Fig. 5B). The large error bar in selenium- group (Fig. 5C) was due to unusually slow growth of one animal in this group. Without inclusion of that animal, the growth curve of selenium- group was the same as the growth curve of the selenium+ group. Vitamin D feeding had very little influence on body weight (Fig. 5D).

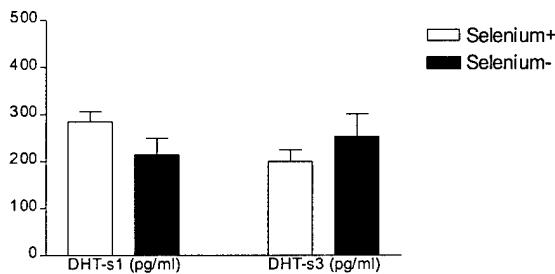
A.



B.



C.



D.

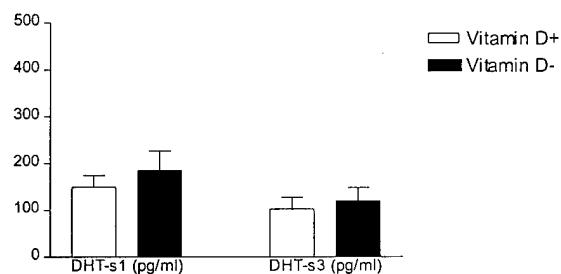


Fig. 6. Serum DHT measurement in animals fed for 1 month (DHT-s1) and for 3 months (DHT-s3) with high fat versus low fat (A), genistein surplus versus deficient (B), selenium surplus versus deficient (C), and vitamin D surplus versus deficient (D). DHT was determined individually in every animal. The DHT levels were measured using the DSL-9600 kit from Diagnostic Systems Laboratories, Inc..

We showed that serum DHT in these animals is around 200 pg/ml (Fig. 6A,B, C, &D), which agrees well with the published data (Rittmaster et al., 1991). The deviation is large in individual animals (Fig. 6A & B), which is expected because the androgen level in serum can fluctuate over a large range. One observation in this experiment shows that the serum DHT appears to be lower in old animals in our experiments.

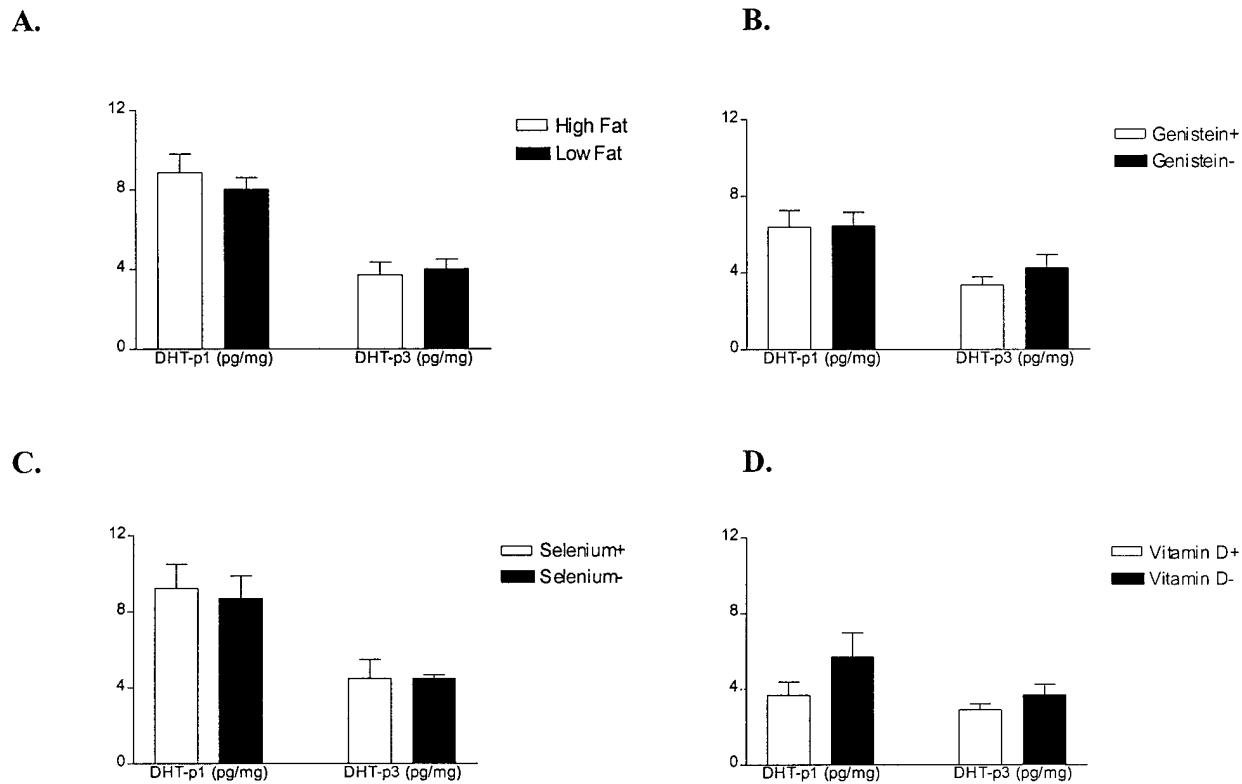


Fig. 7. Intraprostatic DHT measurement in animals fed for 1 month (DHT-p1) and for 3 months (DHT-p3) with high fat versus low fat (A), genistein surplus versus deficient (B), selenium surplus versus deficient (C), and vitamin D surplus versus deficient (D). DHT was determined individually in every left ventral prostate lobe. The DHT levels were measured using the DSL-9600 kit from Diagnostic Systems Laboratories, Inc.. The procedure was essentially the same as recommended by the manufacturer with small modifications.

Intraprostatic DHT ranges from 3 to 9 pg per mg of the wet tissue in the ventral prostate (Fig. 7A, B, C, &D). Our measurement agrees with the results of others (Rittmaster et al., 1991). Similar to the serum DHT levels, the intraprostatic DHT in older animals appears to be lower than that in younger animals in our feeding experiments. There is no significant difference between each pair of animals in these experiments, suggesting that our experimental dietary components have little or no impact on androgen level.

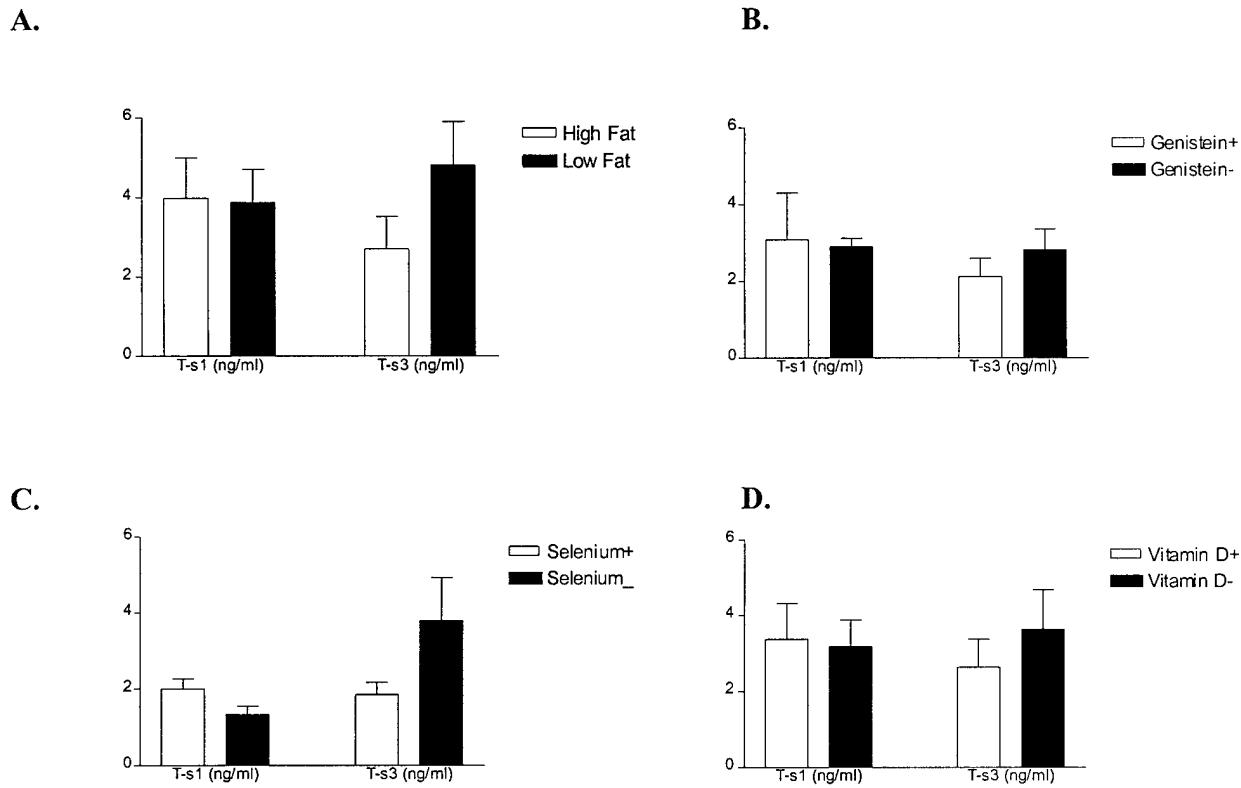


Fig. 8. Serum T measurement in animals fed for 1 month (T-s1) and for 3 months (T-s3) with high fat versus low fat (A), genistein surplus versus deficient (B), selenium surplus versus deficient (C), and vitamin D surplus versus deficient (D). T was determined individually in every animal. The T levels were measured using the DSL-4000 kit from Diagnostic Systems Laboratories, Inc..

The variation of serum T is big. The variation of serum T ranges from 2 to 5 ng/ml (Fig. 8A, B, C, & D). Feeding with high fat diet did not increase the serum T. In contrast, high fat diet caused a reduction in serum T in the 3 month feeding experiment. We plan to do more extensive studies to determine whether this reduction in serum T by high fat diet is statistically significant.

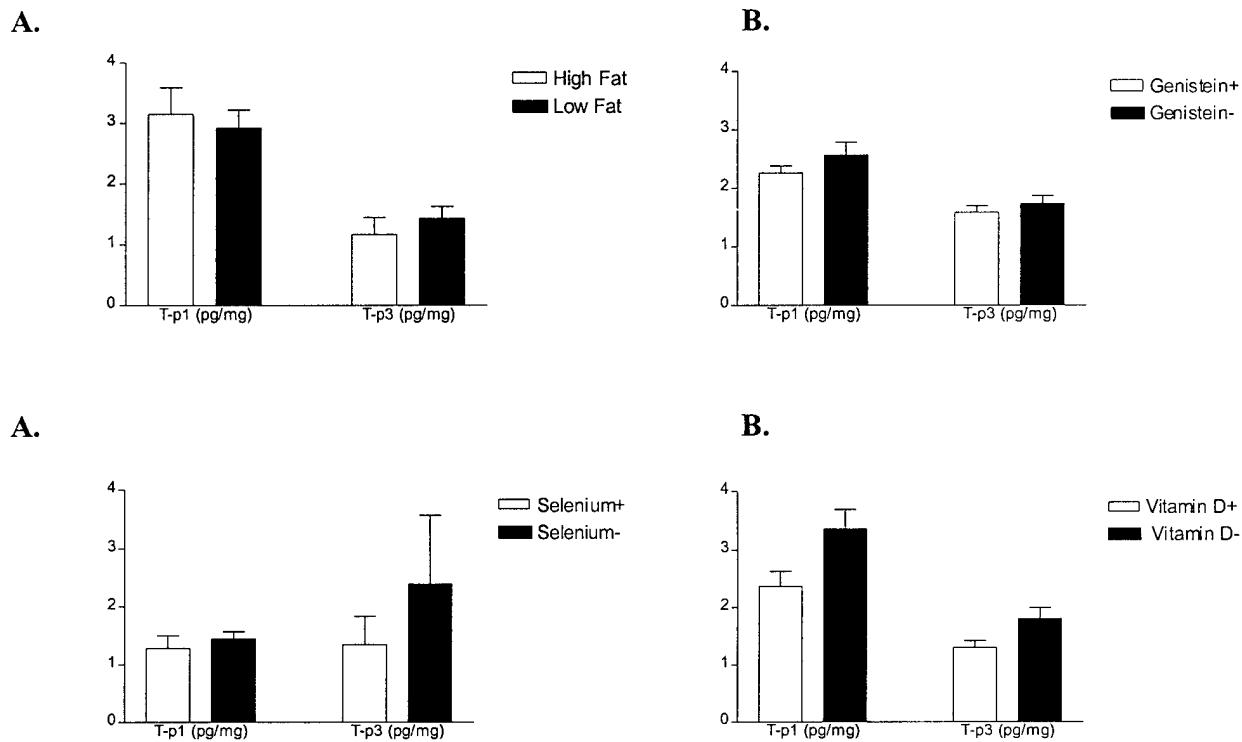


Fig. 9. Intraprostatic T measurement in animals fed for 1 month (T-p1) and for 3 months (T-p3) with high fat versus low fat (A), genistein surplus versus deficient (B), selenium surplus versus deficient (C), and vitamin D surplus versus deficient (D). T was determined individually in every left ventral prostate lobe. The T levels were measured using the DSL-4000 kit from Diagnostic Systems Laboratories, Inc..

The intraprostatic T level variation (Fig. 9A, B, C, & D) is not as dramatic as the variation of the serum T levels. The levels of intraprostatic T levels in the old animals are lower than that in the young animals in our feeding experiments (Fig. 9A, B, & D). The levels of intraprostatic T in the selenium surplus and selenium deficient rats (Fig. 9C) are lower than other feeding groups (Fig. 9A, B, & D).

In the above studies, the differences in the wet weight of the rat ventral prostate between rats fed with high fat diet and rats fed with low fat diet is most significant relative to the effect of other dietary ingredients. More importantly, the fat induced differences in the wet weight of the ventral prostate are consistent in both 1 month and 3 months feeding experiment. Furthermore, dietary fat is the most important risk factor for prostate cancer. Therefore, we decided to further determine the influence of dietary fat on the wet weight of the rat ventral prostate.

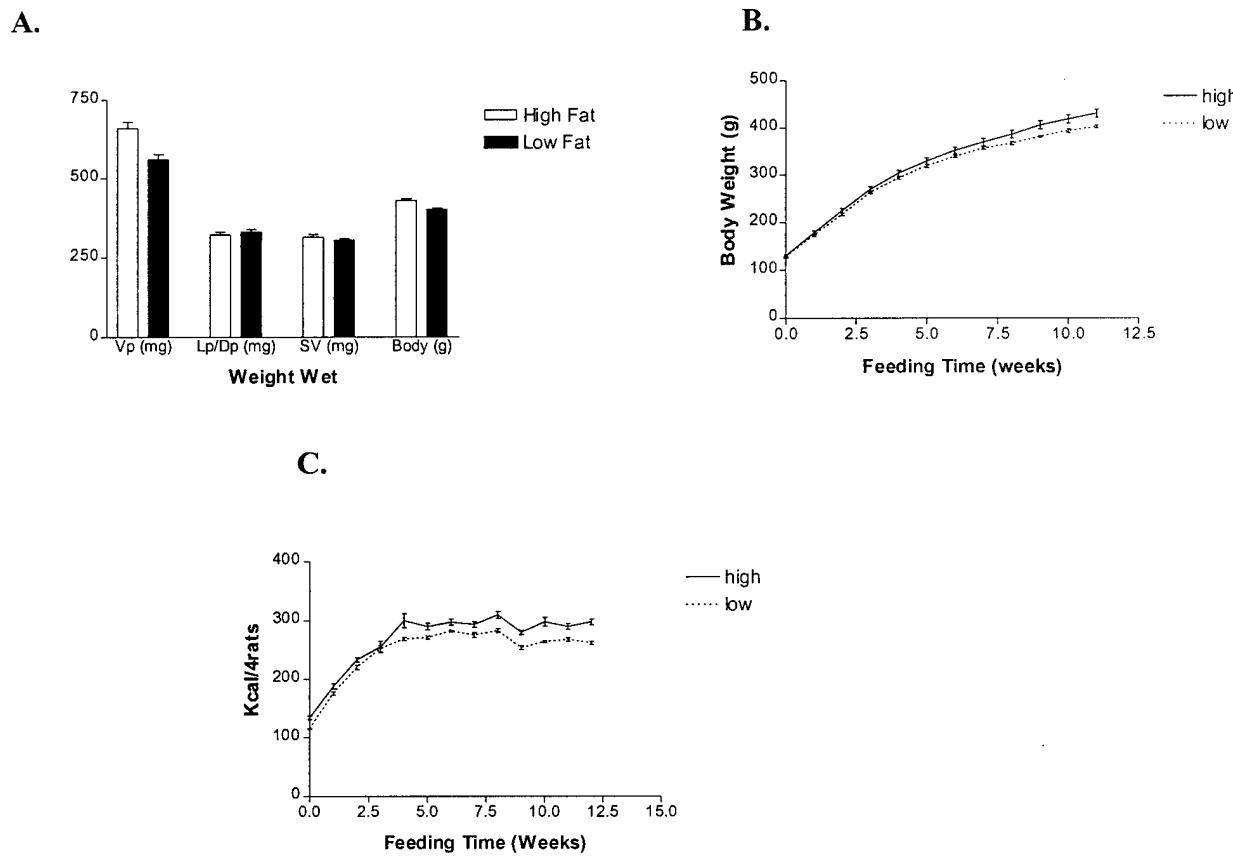


Fig. 10. The effect of dietary fat on the wet weight of the ventral prostate, dorsal-lateral prostate (Dp/Lp), and seminal vesicles and body weight in a large scale animal feeding experiment (A). The growth curve as measured by body weight (B) and energy consumption in the feeding experiment is also illustrated (C).

To unambiguously determine the influence of the dietary fat on the wet weight of the ventral prostate, we performed a large scale feeding experiments using 48 rats. Fig. 9A showed that high fat fed rats developed bigger ventral prostate. The fat had little or no influence on the wet weight of lateral and dorsal prostate and seminal vesicles. The body weight was increased by high fat diet (Fig. 10A & B). The caloric consumption is higher in the high fat fed group than the low fat fed group (Fig. 10C).

Student t-test analysis showed that the difference in the ventral prostate is significant ($p < 0.001$). There is no significant difference in the wet weight of the dorsal-lateral prostate and seminal vesicles. These observations indicate that the dietary fat influence is most dramatic on the ventral prostate, which provides an excellent model to study the molecular mechanism of dietary fat impact on prostate growth.

It is important to point out that dietary fat influence on prostate could involve fat, increased caloric take, or both. These three possible mechanisms can be distinguished by paired feeding, that is, feeding two groups of animals with equal amount of calories everyday. Although fat is considered to be a major risk factor for prostate cancer in men, it is difficult to

rule out the importance of the increased caloric take that is associated with high fat diet. Thus, our results are very much relevant to the dietary fat influence on human.

Task 2. To study the impact of the dietary components on androgen-response gene expression in LNCaP and PC-3 xenograft tumors in nude mice (months 1-30).

We had technical difficulty to initiate this study. So we focused more on Task 1. Because we have shown that diet components in our experiments did not influence androgen-response gene expression in the rat ventral state, we would like to make a change in the research plan.

One extremely important and interesting observation is that the wet weight of the ventral prostate is increased 15% in the rats fed with high fat diet relative to the rats fed with low fat diet. This phenomenon is very significant in the context of the epidemiological data that saturated fat consumption is a major risk factor associated with prostate cancer incidence rate. Furthermore, the ventral prostate can serve as a model to determine the molecular mechanism of fat influence on prostate.

What is the mechanism of fat influence on rat ventral prostate? Our results collectively suggest that the fat influences the prostate via a mechanism independent of androgen axis. Nevertheless, we can hypothesis that the effect of fat diet on the wet weight of the rat ventral prostate is mediated through influencing the expression of certain genes. It is well established that phenotypic changes are always associated with gene expression changes. Thus, 15% increases in wet weight should accompany some gene expression changes. Our lab has the expertise to identify genes that are differentially expressed in two closely related tissues. The identification of these genes would provide insights into the mechanism of fat influence in the prostate.

On the basis of the above discussion, I request to pursue a different Task 2, which was approved by DOD officials.

Task 2 (new). To identify and characterize for genes that are differentially expression in the ventral prostate of the rats fed with high fat diet versus the rats fed with low fat diet.

- a. **To make two PCR-based subtractive cDNA libraries using the same approach as we used before (Wang et al., 1997). One of them is enriched for genes that are up-regulated in the ventral prostate of the rat fed with high fat diet. Another is enriched for genes that are down-regulated in the ventral prostate of the rat fed with high fat diet.**

To make two PCR-based subtractive cDNA libraries, we first purified more than 1 mg total RNA from the ventral prostate of the rat fed with either high fat diet or low fat diet separately. Poly A+ RNA was then selected from the total RNA samples. Double-stranded cDNA synthesis and PCR-based subtraction procedure was essentially the same as described previously. One small modification was introduced to reduce the ratio of the driver to tracer from 20 fold to 10 fold. This adjustment of the driver-tracer ratio would facilitate the enrichment of genes that are less dramatically up or down regulated. We anticipate that the extent of up or down regulation by dietary fat is unlikely to be as extensive as the up or down regulation induced

by androgen manipulation. After repeated subtraction, we have obtained 2 subtractive cDNA libraries that do not cross hybridize with each other. This is a technically challenging and time-consuming procedure.

b. To identify and verify genes that are up- or down-regulated in the ventral prostate of the rats fed with high fat diet relative to the rats fed with low fat diet. Northern blot analysis will be used.

We have screened the PCR-based subtractive cDNA libraries that are enriched for genes potentially up or down regulated by high fat diet. Only 1 gene that is down regulated in the ventral prostate of the rat fed with high fat diet was verified. All of the other candidate genes were proven to be not influenced by high fat diet in the rat. Our lab has extensive experience of conducting PCR-based cDNA subtraction. We have previously demonstrated that this method can identify about half or more of the genes that are differentially regulated between 2 closely related populations of mRNA. The fact that we were only able to identify 1 gene that is down regulated by dietary fat in the ventral prostate (Fig. 11), suggesting that the number of genes differentially regulated by dietary fat is very limited. The extent of down regulation, as demonstrated by the northern blot analysis, was only about 37% (Fig. 12). However, this down regulation is statistically significant ($p < 0.05$). Our finding raises a possibility that dietary fat influences on gene expression in the prostate is very minimal. However, it is possible that these minimal alterations in gene expression could affect the wet weight of the ventral prostate over a long period of feeding time. It will difficult to identify genes that are minimally regulated by dietary fat.

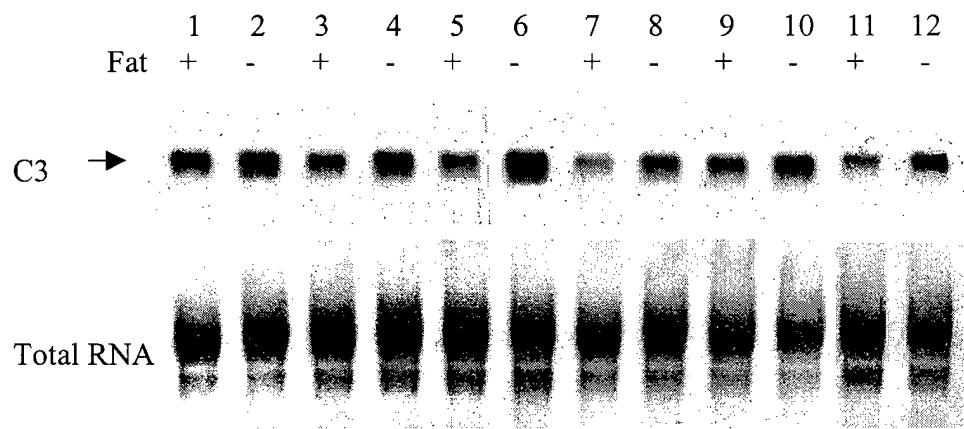


Fig. 11. Identification of a gene that is down-regulated by dietary fat in the rat ventral prostate. Northern blot was used to detect the expression of a gene (indicated by arrow) identified from a PCR-based cDNA subtraction library. Total RNA was isolated from the ventral prostate of rats fed with high fat diet (+) or the rats fed with low fat diet (-) for 3 months. In each lane, the total RNA was isolated from 4 rats and a total of 48 rats were used in this experiment. Total RNA loading was visualized by staining the transferred membrane with methylene blue. The high fat diet contains 30.8% of calories from saturated fat and low fat diet contains 2.3% of calories from fat (See Table 1 for details about dietary contents).

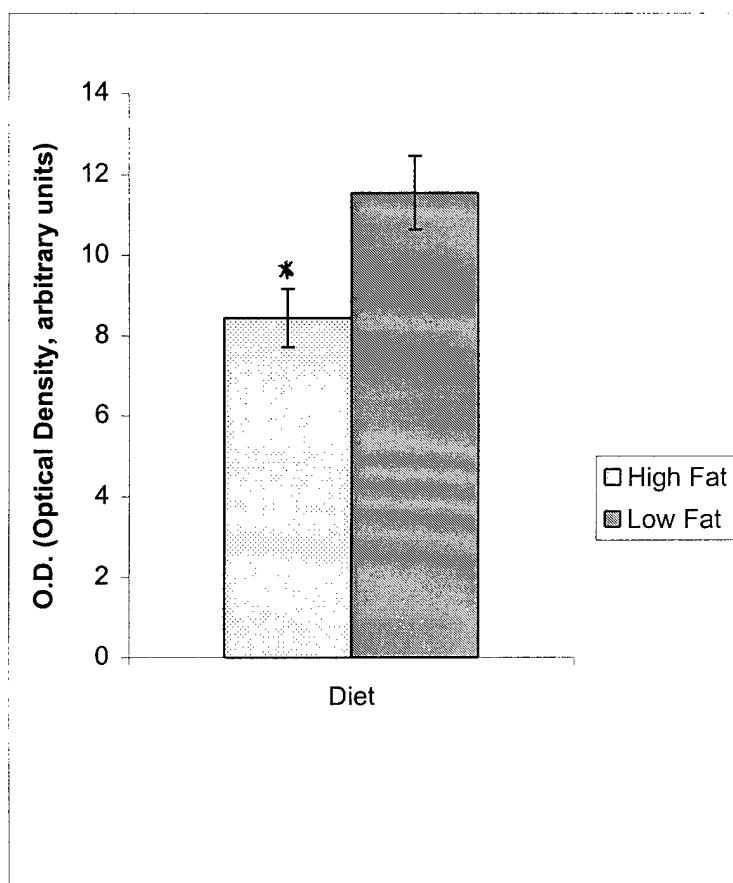


Figure 12. Densitometric analysis of C3 expression in the prostate of high fat vs. low fat fed rats. The northern blot of Fig. 2 is represented in graphical form. Asterisk (*) indicates the statistical significance (Independent t-test, $p < 0.05$).

c. To sequence the cDNA fragments from genes that are up- or down-regulated in the ventral prostate of the rats fed with high fat diet.

We have sequenced the cDNA fragment derived from the gene that is down regulated in the ventral prostate of the rat fed with high fat diet. The sequence matched 100% with the prostatein C3 gene. The prostatein C3 is a ventral prostate-specific secretory protein (Tan et al, 1992). Its major function is involved in secretion. However, it cannot be ruled out the possibility that prostatein C3 can affect the wet weight of the prostate. In our experiments, we have shown that dietary fat only affected the wet weight of the ventral prostate but not the dorsal and lateral prostate in the rat. This observation raised a possibility that dietary fat may affect genes that are ventral prostate specific. Our finding that prostatein C3, a ventral prostate specific protein, is influenced by dietary fat is consistent with this expectation. The functional significance of prostatein C3 in dietary fat action in the prostate remains to be elucidated. The prostatein C3 is an androgen-response gene. However, the expression of other androgen response genes is not influenced by dietary fat. In addition, dietary fat does not influence serum

and intraprostatic androgen levels. Thus, it is likely that the impact that dietary fat on the expression of prostatein C3 is not mediated through the androgen action pathway. It will be interesting to determine the mechanisms by which dietary fat down regulates the expression of prostatein C3, the only known gene that is influenced by dietary fat in the prostate in vivo.

d. To isolate full-length cDNA for genes that are up- or down-regulated in the ventral prostate of the rats fed with high fat diet.

The full-length cDNA for prostatein C3 is available in prostate research community (Tan et al, 1992). Thus, it is not necessary to isolate the prostatein C3 full-length cDNA.

KEY RESEARCH ACCOMPLISHMENTS

1. Dietary fat increases the wet weight of the rat ventral prostate.

The first experiment we performed was to feed rats with diets with defined ingredients. The effect of saturated fat, vitamin D, genistein, and selenium on the wet weight of the ventral, dorsal, and lateral prostate and seminal vesicles were determined. The initial preliminary experiment indicated that dietary fat increased the wet weight of the ventral prostate but not the dorsal and lateral prostate and seminal vesicles. In contrast, the other dietary components did not show demonstrable influences on the wet weight of these tissues.

We next studied more extensively the effect of saturated fat on the wet weight of the ventral prostate. The result confirmed our preliminary observation. After 3 months of feeding, the wet weight of ventral prostate of the high fat fed rats is 660 ± 20 mg (SEM) whereas the wet weight of the ventral prostate of the low fat fed rats is 560 ± 16 mg (SEM). There is a 15% difference between the wet weight of high fat fed rats and that of the low fat fed rats. Statistical analysis with a T-test showed that the difference of the wet weight is significant ($p < 0.001$). In this repeating experiment, we did not find that saturated fat caused any statistically significant differences in the wet weight of dorsal and lateral prostate and seminal vesicles.

This observation is significant because it showed that ventral prostate can serve as a model to determine the molecular mechanism of fat influence on prostate. The observation that only saturated fat but not other dietary ingredients had significant impact on the ventral prostate wet weight is very important when one considers the epidemiological data that saturated fat consumption is a major risk factor associated with prostate cancer incidence rate.

2. Dietary components do not affect the expression of androgen response genes.

We have isolated total RNA from the individual left lobe of the ventral prostate from every group. Each RNA samples were analyzed individually with Northern blot. We have probed the Northern blot filters for the expression of more than 20 androgen-response genes. Although there is slight individual variations in the expression of androgen-response genes, we did not detect any consistent effects of dietary ingredients on androgen-response genes.

3. The effect of dietary ingredients on serum testosterone (T) and dihydrotestosterone (DHT) and intraprostatic T and DHT.

Serum T and DHT were measured using RIA kits. Feeding with various diets did not seem to cause any significant difference in serum T and DHT levels in animals.

We have developed a very convenient method to detect intraprostatic T and DHT, which allowed us to measure the T and DHT in the right lobe of each ventral prostate individually. Similar to the serum T and DHT, feeding with various diets did not seem to cause any significant difference in intraprostatic T and DHT levels in animals.

4. Identification of a gene that is regulated by dietary fat in the ventral prostate of the rat.

Although it is widely accepted that dietary fat plays an important role in the initiation and progression of prostate cancer. The mechanism with which the fat impacts on prostate cancer cells remains unclear. The molecules involved in dietary fat action in the prostate was virtually unknown *in vivo*. Our study represents the first example for genes that are regulated by dietary fat. This gene encodes prostatein C3 and can serve as a model for elucidating the mechanisms by which dietary fat influences gene expression. Also, it will be interesting to determine the function of prostatein C3 in prostate growth.

REPORTABLE OUTCOMES

1. We have worked out a method in the course of our research which allow us to measure the intraprostatic T and DHT in individual rat prostate. This method is being written for submission.
2. Enhancement of rat ventral prostate by high fat diet provides an excellent model system to study the molecular mechanism by which dietary fat influences prostate growth.
3. We have identified a gene that is down regulated by high fat diet in the ventral prostate in the rat. This gene provides a model for elucidating the mechanism by which dietary fat influences gene expression.
4. We have submitted a manuscript entitled “High fat diet increases the weight of rat ventral prostate” for publication. In addition, we are preparing a new manuscript describing the prostatein C3 regulation by dietary fat in the ventral prostate of the rat.

CONCLUSIONS:

We have shown that fat, genistein, vitamin D, and selenium in diet had little or no influence on the expression of androgen-response genes in the ventral prostate of rats. The dietary components in our experiments had little or no effect on serum T, intraprostatic T, serum DHT, and intraprostatic DHT.

One exciting observation is that dietary fat increased the size of the ventral prostate by **10-15%** and this increase is statistically significant. However, dietary fat had little or no influence on the size of dorsal-lateral prostate and seminal vesicles. This result shows that fat can impact on the growth of prostate either directly or indirectly. Our discovery shows that fat

impact on the ventral prostate provides an excellent model to study the molecular mechanism of dietary fat influence in the prostate.

Genes that are up or down regulated by dietary fat were assessed by using a highly sensitive PCR-based cDNA subtraction method. We have developed this method and have successfully applied this approach to identified genes that are differentially expressed in two closely related tissues. The finding that prostatein C3 is down regulated by dietary fat in the ventral prostate of the rat *in vivo* is a major advancement in elucidating the mechanisms by dietary fat influences prostate growth because this represents the first example of genes that are regulated by dietary fat *in vivo* in the prostate.

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APPENDICES:

One manuscript entitled: "High fat diet increases the weight of rat ventral prostate" is attached.

BIBLIOGRAPHY AND PERSONNEL REPORT:

One manuscript entitled: "High fat diet increases the weight of rat ventral prostate" has been submitted and another one entitled: "High fat diet down-regulates the expression of prostatein C3 in the rat ventral prostate" is in preparation.

Personnel	Effort	Period
Zhou Wang	15%	09/01/98 – 01/31/01
John T. Grayhack	5%	09/01/98 – 01/31/01
James M. Kozlowski	5%	09/01/98 – 01/31/01
Xiaoyan Cai	100%	09/01/98 – 01/31/01
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High Fat Diet Increases the Weight of Rat Ventral Prostate

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Running Title: Dietary Fat Increases Prostate Weight

Key Words: Dietary fat; rat ventral prostate; androgen

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Abstract

BACKGROUND. Understanding the mechanisms by which diet influences the prostate may eventually lead to novel approaches for preventing prostate cancer. The objective of this research is to examine the impact of dietary fat, vitamin D, and genistein on prostate weight, serum and intraprostatic androgen levels, and the expression of several androgen-response genes.

METHODS. Sprague-Dawley rats were fed, beginning at 21 days of age, for 1 or 3 months of experimental diets with high saturated fat (32.2% calories from fat), low saturated fat (3.6% calories from fat), genistein plus (20 mg/kg), genistein deficient, vitamin D surplus (4,000 U/kg), or vitamin D deficient. The body weight, food intake, the weights of the ventral prostate and dorsolateral prostate, and the levels of testosterone and dihydrotestosterone (DHT) in serum and in the prostate were determined. The expression of androgen-response genes was characterized by Northern blot analysis.

RESULTS. The pilot experiments showed that high dietary fat appeared to consistently increase the weight of the ventral prostate, while vitamin D or genistein did not have a consistent effect on prostate weight. Further analysis confirmed that the ventral prostate is 15% ($p < 0.001$) heavier in the rat on a high fat diet as compared to a low fat diet. Dietary fat had no significant influence the levels of serum and intraprostatic androgens and the expression of androgen-response genes.

CONCLUSIONS. Our results suggested that the ventral prostate weight of the rat is increased, without affecting the androgen axis, by feeding the animals with high fat diet beginning at 21-days of age. This observation is potentially important since epidemiological data suggest that saturated fat consumption is a major risk factor associated with prostate cancer incidence rate.

Introduction

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer death in men in the United States [1]. The incidence of clinically significant prostate cancers varies dramatically in different regions of the world [2]. Asian countries have a much lower rate of prostate cancer incidence than Western countries. Autopsy studies of men who died for reasons other than prostate cancer showed that approximately 60% of all men have latent or clinically silent prostate tumor [3]. Although the rate of latent cancer is similar between the U.S. and Japan, the clinical prostate cancer incidence in the U.S. is much higher than that in Japan. Nutrition and lifestyle are thought to be responsible for the low rate of clinical prostate cancer incidence in Japan, because the incidence of clinically detected prostate cancer in Japanese men increases within one generation after migration to the U.S. [4, 5]. It is believed that the Asian diet is associated with a low prostate cancer rate whereas the Western diet is associated with a high prostate cancer rate. Nutrition appears to be a major risk factor responsible for the differences in global distribution of clinical prostate cancer.

Human and animal studies indicate that certain dietary ingredients can modulate the growth rate of prostate cancer cells [6-10]. The risk of developing clinically significant prostate cancer appears to be affected by dietary fat intake, fatty acids, obesity, dietary fiber intake, fruits, vegetables, antioxidants, and soy protein intake. Considering that androgen is the most potent mitogen for prostatic cells *in vivo*, it has been suggested that the impact of some dietary components on the prostate cancer development and progression may involve the androgen action axis [11].

Dietary components included in this study, saturated fat, vitamin D, and genistein, may affect androgen action in the prostate. The testosterone level has been reported to be higher in

men on a high-fat diet than men on a low-fat diet [12]. Also, certain unsaturated fatty acids could inhibit 5 α -reductase, an enzyme that converts testosterone to DHT [13], leading to the possibility that dietary fat may influence androgen action axis.

1,25-Dihydroxyvitamin D is inhibitory to the proliferation of some human prostate cancer cells [14-16]. Vitamin D may influence the androgen action pathway since the vitamin D receptor (VDR) and androgen receptor (AR) belong to the same superfamily of ligand-dependent nuclear receptors. Vitamin D has been shown to up-regulate the expression of AR and prostate-specific antigen in androgen sensitive LNCaP human prostate cancer cells [17, 18]. Thus, vitamin D may modulate the expression of androgen-response genes in the prostate *in vivo* via modulation of the AR expression.

Soy-based diets, rich in the isoflavones genistein and daidzein, appear to protect against prostate cancer [19, 20]. Genistein is inhibitory to AR-dependent transactivation in the presence of androgen [21]. Genistein also has a weak estrogenic effect [22], possibly due to an interaction with the estrogen receptor(s). Furthermore, genistein and related isoflavones are metabolized by the liver using metabolic pathways similar to those for metabolizing sex hormones. The above information suggests that genistein could also influence androgen-response gene expression in the prostate.

Understanding the mechanism by which dietary components influence the normal prostatic cells *in vivo* will provide insights into the impact of dietary components in prostate cancer initiation and/or progression. While much has been done with studying dietary compounds in cell lines *in vitro*, little work has been done *in vivo*. The present paper describes the effect of dietary components, particularly saturated fat, on the prostate and androgen action axis using the rat as a model.

Materials and Methods

Animals

A total of 120 male Sprague-Dawley rats at age of 21 days were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN) via the Northwestern University Animal Care Facility, which is accredited by AAALAC. Seventy-two rats were used in the pilot experiment and the rest in the confirmatory experiment. Upon arrival, the rats were segregated into groups of 4 rats randomly that were housed as a group in individual cages. Each cage was provided with 1 of the 6 diets, high or low fat, vitamin D supplement added or deficient, genistein supplement added or absent. Animals were weighed once a week and fed twice a week. The animals were sacrificed by thoracotomy under methoxyflurane anesthesia after 4 or 12 weeks of experimental diet exposure. The blood was collected via cardiac puncture for serum testosterone and dihydrotestosterone (DHT) determination. The ventral and dorsolateral lobes of the prostate were dissected as previously described [23]. Seminal vesicles weights were measured after serial blotting of the incised vesicle to remove the seminal vesicle fluid. After dissection, the prostate lobes were immediately frozen in liquid nitrogen for RNA isolation and measurement of testosterone and DHT levels in the prostate.

Diets

Six experimental diets were prepared by Harland Teklad Inc. (Madison, WI). As illustrated in Table 1, the experimental diets are similar in composition of most constituents. To compensate for the known observation that animals on a low fat diet consume more food than those on a high fat diet, the composition of the low fat diet was modified so that the total calories

per gram of food in the lower fat groups were less than those in the high fat group. The food consumption was determined by weighing the leftover food in each cage. Total weekly caloric consumption per cage of animals was calculated from the amount of the consumed food. Fat calories from the hydrogenated coconut oil were balanced with carbohydrate calories from sucrose. Since 4 rats were housed in each cage, a pair-feeding procedure was not attempted. The diets were aliquoted and stored in at -20 C freezer.

Measurement of Serum and Intraprostatic Androgens

The blood samples collected by cardiac puncture were centrifuged at 2500 rpm for 5 min in a clinical centrifuge to obtain serum. Serum samples were stored at -80 C and were measured for testosterone and DHT levels individually. The intraprostatic testosterone and DHT were measured in the left ventral prostate lobe individually. Each ventral lobe was homogenized at 80 mg/ml in a buffer consisting of 1 x PBS, 100 mM EDTA, 100 uM PMSF, 10 uM leupeptin, 1 uM pepstatin, and antifoam B emulsion (150 ul per 50 ml of the buffer) using a tissue homogenizer (Ultra-Turrax T25) at top speed. The homogenized samples were centrifuged at 3700 rpm for 20 min at 4 C and the supernatants collected for androgen measurement. Testosterone and DHT levels in serum or in prostate homogenate were determined using the DSL-4000 kit and DSL-9600 kit from Diagnostic Systems Laboratories, Inc., respectively. For the DSL-4000 kit, the co-efficiency of intra-assay variation is 7.9 – 9.6% and the co-efficiency of inter-assay variation is 8.4 – 9.1%. For DSL-9600 kit, the co-efficiency of intra-assay variation is 3.1 – 6.2% and the co-efficiency of inter-assay variation is 2.3 – 8.5%.

RNA isolation and analysis

Total RNA was isolated from the right ventral prostate lobe using the guanidinium/CsCl gradient method [24]. The RNA was analyzed by electrophoresis on a 1% agarose-formaldehyde gel and 10 ug of the total RNA was loaded in each lane. After electrophoresis, the RNA was transferred onto nylon membrane for Northern blot analysis for the expression of various androgen-response genes as previously described [25].

Statistical Analysis

All the data were calculated using GraphPad Prism by GraphPad Software Incorporated. Results were expressed as the means \pm S.E.M.. The statistical significance of differences in the data was evaluated using student t-test (SPSS statistic software package). The *p* values less than 0.05 were considered significant and less than 0.01 very significant.

Results

Pilot studies on the effect of dietary fat, vitamin D, and genistein.

The feeding of experimental diets was carried out using a total of 72 male Sprague-Dawley rats and the feeding was initiated when the rats were at the age of 21 days. Fig. 1A and Fig. 1B show the results of the pilot feeding experiment over 1 and 3 month periods, respectively. A total of 24 rats were used in the 1 month feeding experiment with 4 animals on each experimental diet. A total of 48 rats were used in the 3 month feeding experiment with 8 animals on each experimental diet. The body weight and the wet weight of ventral prostate, dorsolateral prostate, and seminal vesicles were determined at the end of the feeding experiments. Seminal vesicles were squeezed with forceps to eliminate seminal fluid prior to weighing.

The ventral prostate of the rats fed with a high fat diet is slightly larger than that of the rats fed with a low fat diet in the 1 month feeding experiment (Fig. 1A). The increase in the ventral prostate wet weight was also observed in the 3 month feeding experiment (Fig. 1B) and the ventral prostate difference between the high-fat and low-fat groups appears to be more significant after the 3 months feeding. The weight of the seminal vesicles also appeared to be increased by the high fat diet in both the 1 month and 3 month feeding experiments. The weight of the dorsolateral prostate was not consistently affected by the high fat diet in the 1 month and 3 month feeding.

Vitamin D had little or no effect on prostate size in the 1 month and 3 month feeding experiments (Fig. 1A & 1B). One observation of this experiment is that the size deviation of the ventral prostates of the rats fed with a vitamin D deficient diet is larger than the deviation in other groups. This may imply the importance of vitamin D in maintaining normal prostate

structure and function. No consistent effect by vitamin D on the size of the dorsolateral prostate or seminal vesicles was observed.

Genistein appeared to cause a small reduction in the size of the ventral prostate, dorsal and lateral prostate, and seminal vesicles in the 1 month feeding experiment (Fig. 1A).

However, the small reduction was not observed in the dorsolateral prostate and seminal vesicles in the 3 month feeding (Fig. 1B). The size of the ventral prostate by genistein was only reduced very slightly in the 3 month feeding experiment (Fig. 1B), suggesting that the influence of genistein feeding on prostate weight was minimal.

The body weight of the rats fed with the high fat diet is slightly heavier than the rats on the low fat diet (Fig. 1A and 1B) and was statistically significant in the 1 month feeding experiment ($p = 0.029$) but not in the 3 month feeding experiment ($p = 0.217$). The slight increase in the body weight is most likely due to the accumulation of the fat that can be seen obviously in the body cavity of the dissected animals. Vitamin D and genistein in the diet did not influence the body weight (Fig. 1A and 1B).

Dietary fat increases weight of the rat ventral prostate.

In the pilot studies, the differences in the wet weight of the ventral prostate between rats fed with the high fat diet and rats fed with the low fat diet were more obvious relative to the effect of other dietary ingredients. Also, the fat induced differences in the wet weight of the ventral prostate were consistent in both the 1 month and 3 month feeding experiments. Furthermore, dietary fat appears to be one of the most important risk factors for prostate cancer [9]. Therefore, we further studied the influence of dietary fat on the wet weight of the rat prostate.

We performed a feeding experiment using 48 rats with half of them fed with a low fat diet and another half with a high fat diet for 3 months. Fig. 2A showed that the rats fed with high fat developed heavier ventral prostates than the rats on the low fat diet ($p < 0.001$). The fat had no significant influence on the wet weight of the dorsolateral prostate ($p = 0.403$) and seminal vesicles ($p = 0.626$). The body weight was increased by the high fat diet (Fig. 2A & B) ($p < 0.001$), and the caloric consumption is slightly higher in the high fat fed group than the low fat fed group ($p < 0.001$) (Fig. 2C). These observations argue that the influence of dietary fat on the rat prostate is most evident on the ventral lobes.

The effect of dietary fat on androgens

To determine the impact of dietary fat on androgen action, we have measured the androgen levels in serum and prostate samples collected in the confirmatory feeding experiment. The androgen levels were determined in each rat individually. No statistically significant changes were detected in serum testosterone ($p = 0.119$), serum DHT ($p = 0.440$), intraprostatic testosterone ($p = 0.586$), and intraprostatic DHT ($p = 0.612$) between the animals fed with the high fat and low fat diets (Fig. 3A, B, C & D).

The effect of diet on the expression of androgen-response genes in the prostate

To further study the possible dietary impact on the androgen axis in the prostate, we examined the effect of dietary fat on a few androgen-response genes by extracting the total RNA from the pooled left lobes of the ventral prostates of the rats fed with the same diet. The RNA was analyzed by Northern blot using cDNAs of various androgen-response genes as probes. The androgen-response genes tested include farnesyl pyrophosphate synthase (FPPS), calreticulin

(CRT), spermidine synthase (Spd-S), and a novel up-regulated androgen-response gene U19 [25]. These genes were chosen because they respond to androgen manipulation rapidly and dramatically [25]. The Northern blot of FPPS, CRT, U19, and Spd-S are shown in Fig. 4. The high fat diet did not seem to alter the expression level of these androgen-response genes. GAPDH expression pattern and total RNA staining were included as controls for loading normalization. Our results suggest that the dietary fat in our experiment had little or no influence on the expression of androgen response genes.

Discussion

In the present study we have examined the influence of dietary fat, vitamin D, and genistein on the prostate using the rat model. The above dietary components were chosen because they were implicated as risk factors for the incidence rate of prostate cancer [15]. Elucidating the influences of these dietary components on the normal prostate will provide insights into the mechanisms by which these important dietary ingredients modulate the initiation and/or progression of prostate cancer.

Our pilot studies showed that dietary fat could influence the size of the prostate, particularly the ventral prostate, and the influence was consistent in both 1 month and 3 month feeding experiments. In contrast, the influence of vitamin D and genistein on the size of the prostate was very small and was not consistently observed in the 1 month and 3 months feeding experiments. These observations have led us to focus on the effect of dietary fat on the prostate. In the confirmatory experiment, we observed a statistically very significant increase ($p < 0.001$) in the wet weight of the ventral prostate in the rats on the high fat diet relative to that on the low fat diet.

The 15% increase in the wet weight of the normal rat ventral prostate to high fat diet seems to be less dramatic than the response of prostate tumors to the fat diet. Previous studies reported that xenograft prostate tumors in nude mice fed with a high fat diet were about twice in size relative to that in mice fed with a low fat diet [26]. This dramatic size increase in xenograft prostate tumor is likely to be associated with excessive proliferation. The lack of dramatic size enlargement in the normal prostate in response to high dietary fat may imply the existence of a mechanism that restricts the growth of the normal prostate. In contrast, the proliferation of

prostate cancer cells may not be subject to such a restrictive mechanism. Thus, the influence of dietary fat on prostate tumor could be very different from that on the normal prostate.

The mechanism by which dietary fat influences the prostate is unclear. As suggested previously, one possibility is that dietary fat can modulate androgen action in the prostate [11, 12]. To explore this potential mechanism of dietary fat action in the prostate, we have measured the testosterone and DHT levels in both serum and prostate individually in each animal. No statistically significant difference was detected in androgen levels between the rats fed with a high fat diet and the rats fed with a low fat diet. Furthermore, no significant difference in the expression of certain androgen-response genes was observed between these rats. Our results suggest that the influence of dietary fat on the prostate is not mediated by altering androgen action pathway.

In summary, our study did not find statistically significant influences of dietary fat on serum and intraprostatic androgen levels and the expression of a subset of androgen-response genes in the prostate. One statistically very significant influence on the prostate by diet is the 15% weight increase in the ventral lobe of the rats on a high dietary fat. This observation is important if one considers the epidemiological data that saturated fat consumption is a major risk factor associated with increased prostate cancer incidence rate. The rat ventral prostate provides a model to study the molecular mechanism by which dietary fat influences the normal prostate. Understanding such mechanism may lead to the development of novel approaches for preventing prostate cancer because high dietary fat consumption in Western diet appears to associate with prostate cancer progression.

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Figure and Table Legends

Fig. 1. The effect of high fat versus low fat, vitamin D surplus versus deficient, genistein surplus versus deficient on the wet weight of ventral prostate (Vp), dorsolateral prostate (DLp) and seminal vesicles, and the body weight. The feeding was initiated when animals were 21 days old and fed with the experimental diets. A. The animals were fed for 1 month and 4 rats were used in each feeding condition. The body weight difference between rats on the high fat diet and rats on the low fat diet was the only difference with statistical significance ($p = 0.029$) in the 1 month feeding experiment and was marked by (*). B. The animals were fed for 3 months and 8 rats were used in each feeding condition. No difference was statistically significant in the 3 month feeding experiment. The data were analyzed using the GraphPad and SPSS softwares. The error bars represent standard error mean (S.E.M.).

Fig. 2. A. The effect of dietary fat on the wet weight of the ventral prostate (Vp), dorsolateral prostate (DLp), and seminal vesicles (SV), and the body weight. The difference in the ventral prostate or body weight between high fat and low fat group is statistically very significant ($p < 0.001$) and marked by (*). No statistically significant difference was detected in the dorsolateral prostate and seminal vesicles between the high fat and low fat groups. B. The growth curve of the animals fed with high fat or low fat diet as measured by the body weight. C. The energy consumption in the feeding experiment. A total of 48 rats were used in this experiment with 24 on high fat diet and the other 24 on low fat diet. The feeding were carried out for 3 months and started when the animals were at 21 days of age. The data were processed using the GraphPad and SPSS softwares and the error bars are standard error mean (S.E.M.).

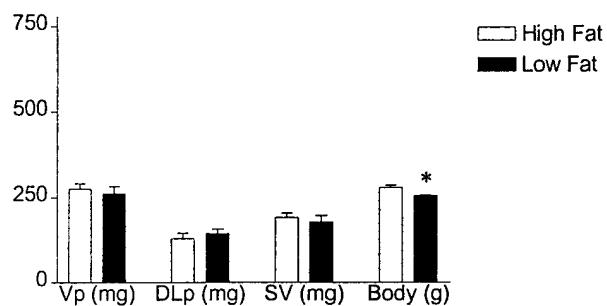
Fig. 3. The effect of dietary fat on serum DHT, serum testosterone (T), intraprostatic DHT, and intraprostatic T. The animals were the same as described in Fig. 2. The levels of androgens were measured for each rat individually. The data collected were processed using the GraphPad and SPSS softwares and the error bars represent standard error mean (S.E.M.).

Fig. 4. The effect of dietary fat on the expression of androgen-response genes in the rat ventral prostate. The animals were fed with either high fat diet (+) or low fat diet (-) for 3 months and the total RNA was extracted from individual ventral prostate. Northern blot was used to detect the expression of the indicated androgen-response genes. FPPS = farnesyl pyrophosphate synthase; CRT = calreticulin; U19 = up-regulated gene 19 (no homology with known genes); Spd-S = spermidine synthase; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; RNA = total RNA stained with methylene blue.

Table 1. Ingredients of experimental diets are indicated in grams (g). AIN-76TM vitamin mix and AIN-76TM salt mix were prepared as described previously [27]. * = "Vitamin-Free" casein. # = a modified AIN-76TM vitamin mix that lacks vitamin D. Genistein is synthetic from Sigma (Catalog#: G 6649). The dietary components that were different between the high fat and low fat, vitamin D surplus and deficient, or genistein surplus and deficient were bolded. Since the animals on a lower fat diet were expected to consume more food than those on a higher fat diet, the caloric density in the lower fat group (3.57 kcal/g) was less than that in the high fat group (4.21 kcal/g). The fat calories from hydrogenated coconut oil were balanced with sucrose.

FIGURE 1

A.



B.

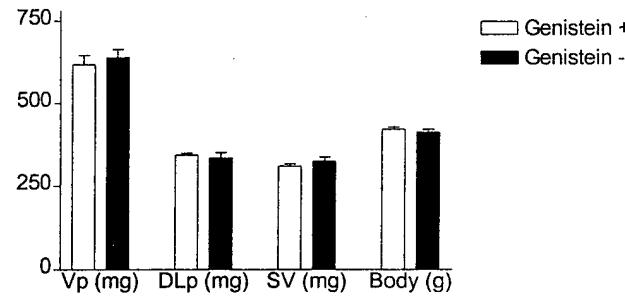
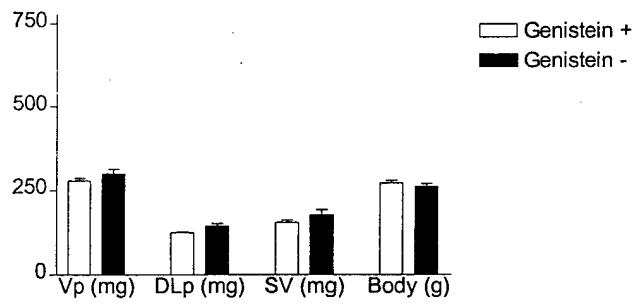
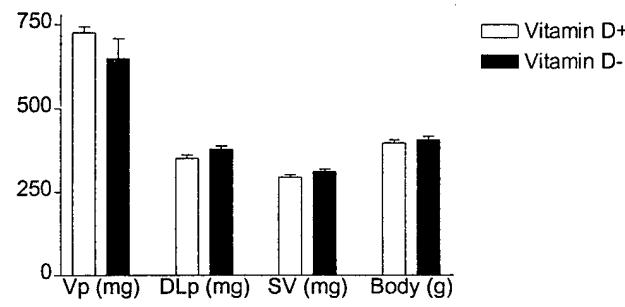
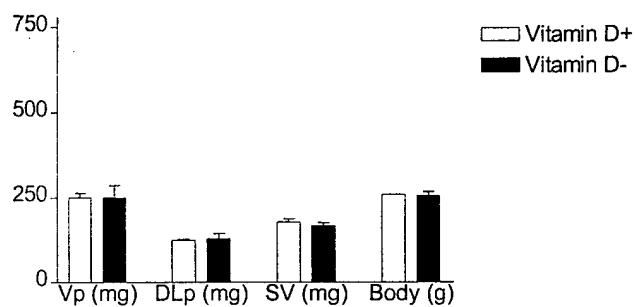
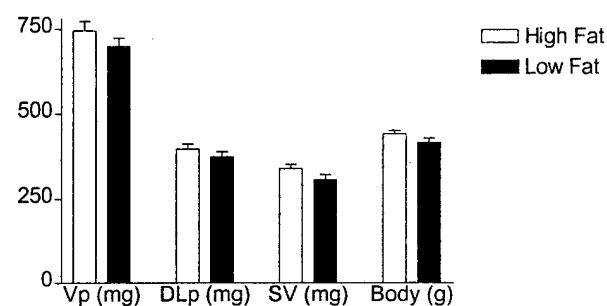
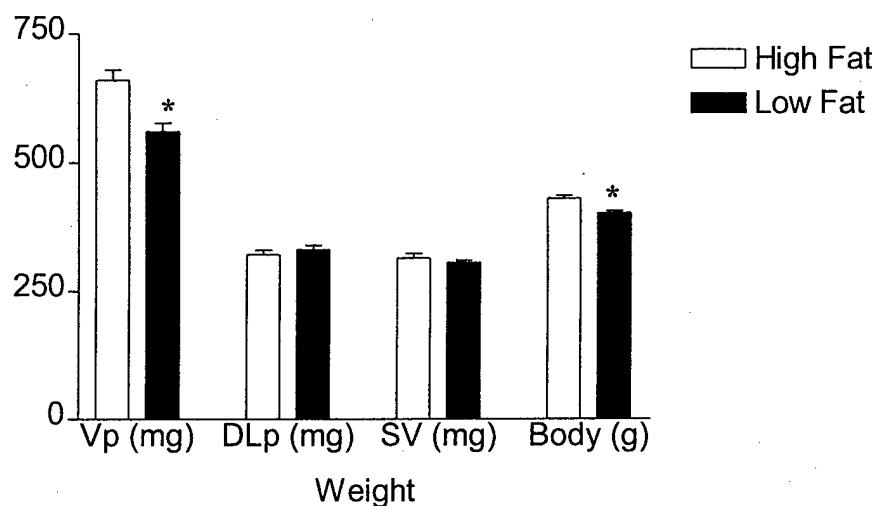
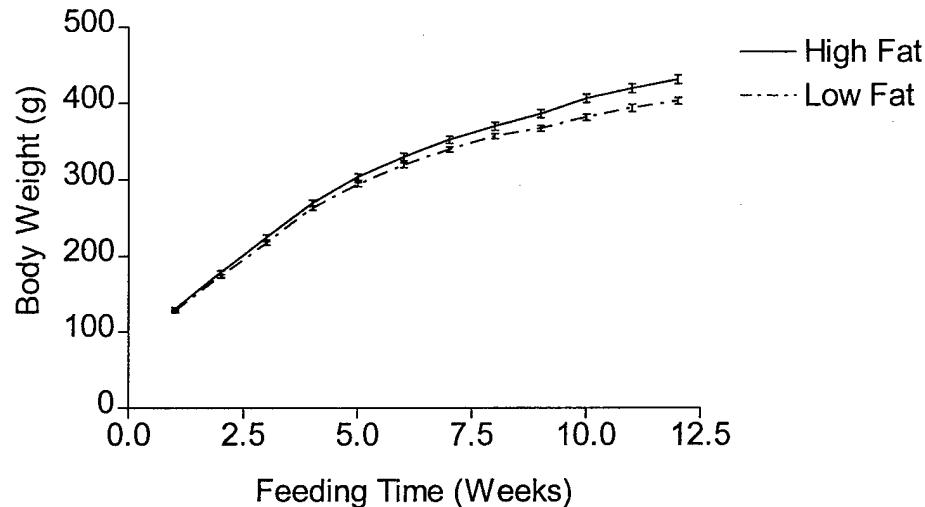


FIGURE 2

A.



B.



C.

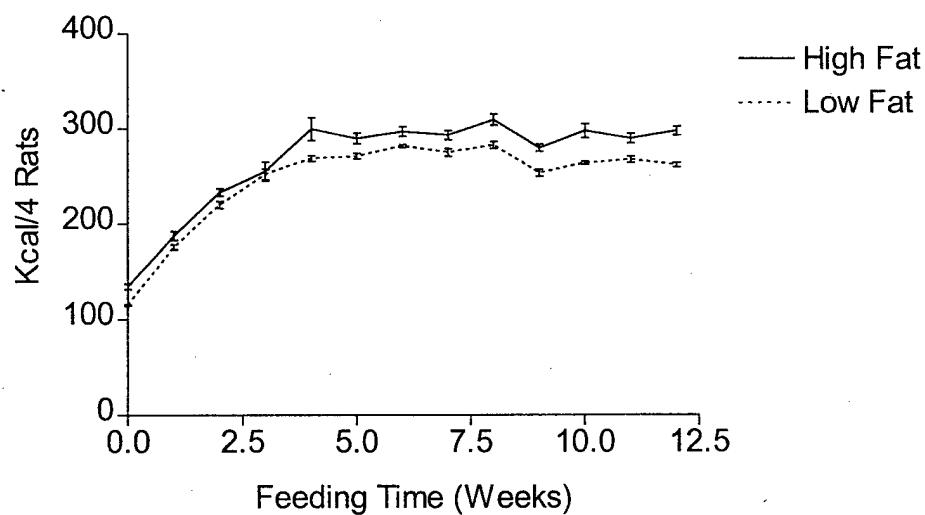


FIGURE 3

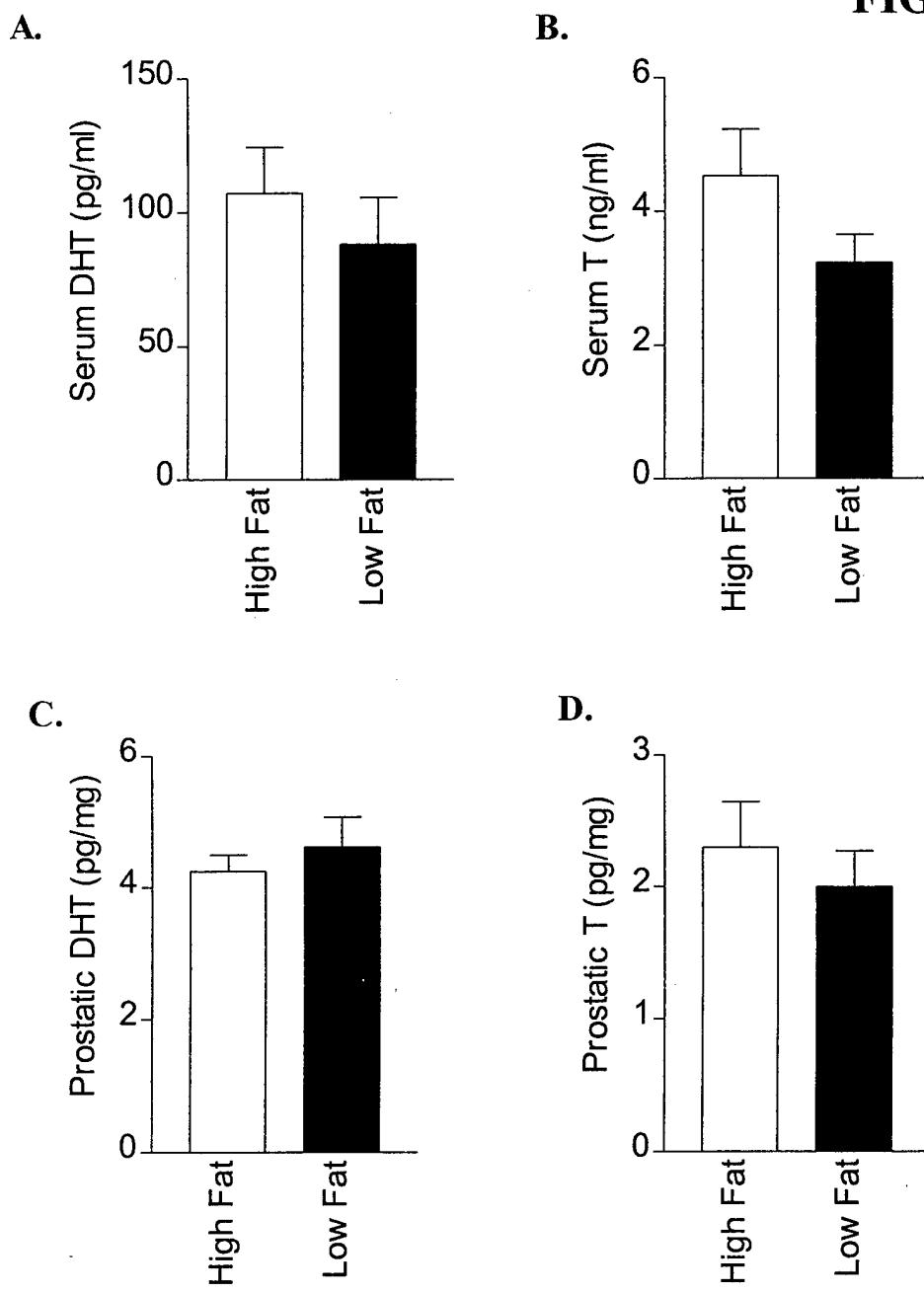


FIGURE 4

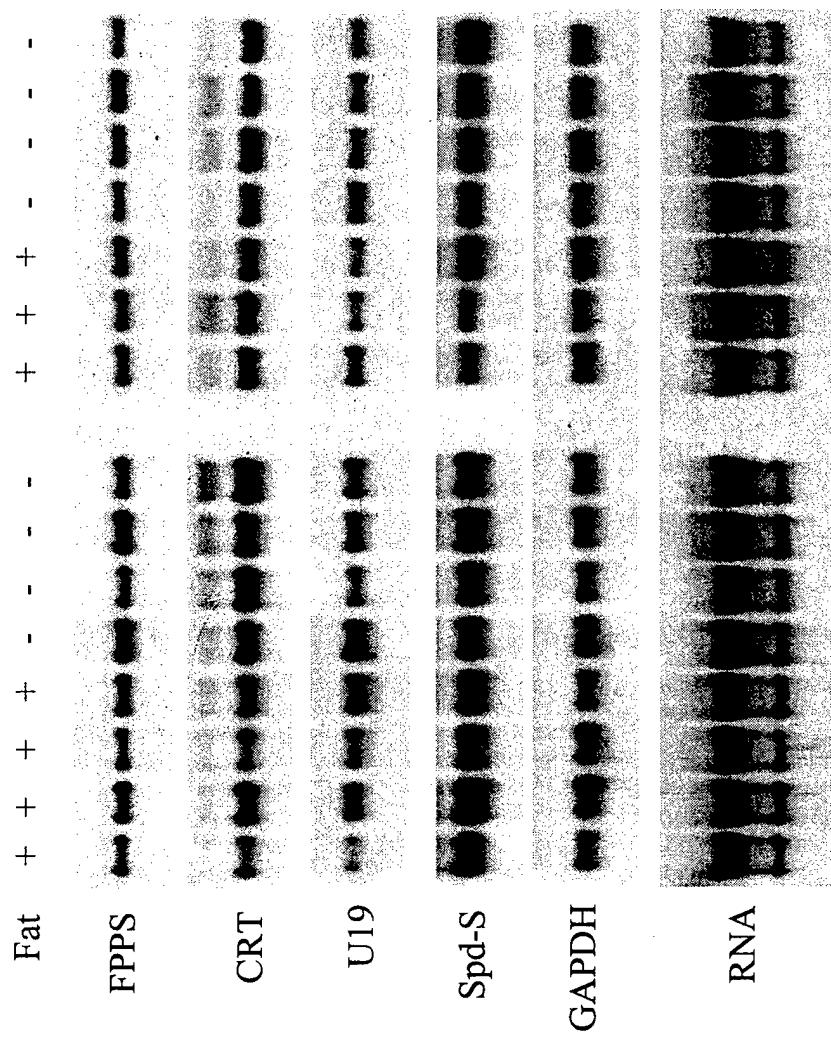


Table 1.

Ingredient	Fat		Vitamin D		Genistein	
	+	-	+	-	+	-
Casein	230	200	196*	196*	200	200
Maltodextrin-10	57.5	50	50	50	50	50
Cornstarch	115	100	100	100	100	100
AIN-76™ vitamin mix	11.5	10	10 [#]	10 [#]	10	10
DL-Methionine	3.45	3	3	3	3	3
Cellulose	50	50	50	50	50	50
AIN-76™ salt mix	40.25	35	35	35	35	35
Choline bitartrate	3.7	3.7	2	2	2	2
Calcium Carbonate	2.3	2	2	2	2	2
Corn oil	12	12	50	50	50	50
Vitamin D3 (400,000U/g)	0	0	0.01	0	0	0
Genistein	0	0	0	0	0.02	0
Sucrose	338.3	534.3	502	512	498	498
Hydrogenated coconut oil	136	0	0	0	0	0
Kcal/g	4.21	3.57	3.77	3.77	3.76	3.76